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### Authors

Walunas, TL  
Bakker, CY  
Bluestone, JA

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# CTLA-4 Ligation Blocks CD28-dependent T Cell Activation

By Theresa L. Walunas, Christina Y. Bakker, and Jeffrey A. Bluestone

*From the Committee on Immunology, Department of Pathology and the Ben May Institute for Cancer Research, The University of Chicago, Chicago, Illinois 60637*

## Summary

CTLA-4 is a CD28 homologue believed to be a negative regulator of T cell function. However, the mechanism of this downregulatory activity is not well understood. The present study was designed to examine the effect of CTLA-4 ligation on cytokine production, cell survival, and cell cycle progression. The results demonstrate that the primary effect of CTLA-4 ligation is not the induction of apoptosis. Instead, CTLA-4 signaling blocks IL-2 production, IL-2 receptor expression, and cell cycle progression of activated T cells. Moreover, the effect of CTLA-4 signaling was manifested after initial T cell activation. Inhibition of IL-2 receptor expression and cell cycle progression was more pronounced at late (72 h) time points after initial activation. The effects of anti-CTLA-4 mAbs were most apparent in the presence of optimal CD28-mediated costimulation consistent with the finding that CTLA-4 upregulation was CD28-dependent. Finally, the addition of exogenous IL-2 to the cultures restored IL-2 receptor expression and T cell proliferation. These results suggest that CTLA-4 signaling does not regulate cell survival or responsiveness to IL-2, but does inhibit CD28-dependent IL-2 production.

CD28/B7 interactions play a central role in providing costimulatory signals to T cells. Ligation of CD28 by B7-1 and/or B7-2 has been shown to induce T cells to regulate growth factor (IL-2) production (1, 2), T helper cell differentiation (3, 4), and programmed cell death (5). Over 8 yr ago, a structural homologue of CD28, CTLA-4, was discovered among a set of T cell-specific, activation-induced genes (6). Not only was CTLA-4 found to share structural homology with CD28, but it also shares the ability to bind B7-1 and B7-2 (7, 8). Further studies demonstrated that the CTLA-4 glycoprotein is expressed on the surface of activated CD4<sup>+</sup> and CD8<sup>+</sup> T cells (9–11) and on activated B cells (12). Whereas CD28 is constitutively expressed on resting human and murine T cells (13), CTLA-4 is upregulated after T cell activation, with peak expression between 48–72 h (9, 10). Initial studies have also suggested that CTLA-4 upregulation is CD28-dependent. First, anti-CD28 mAbs accelerate the kinetics of CTLA-4 mRNA accumulation in human PBL (14). Second, the degree of CTLA-4 upregulation was significantly reduced on T cells from CD28-deficient mice (10).

Early findings suggested that CTLA-4 ligation on activated T cells provided additional costimulatory signals (9). However, recent studies in vitro (10, 11) and in vivo (15) have suggested that rather than acting to costimulate T cells, CTLA-4 may actually be involved in the downregulation of T cell responses, perhaps as an antagonist of CD28 costimulation. Monovalent Fab fragments of an anti-murine CTLA-4 mAb, which prevent the interaction of CTLA-4 with its counter-receptors, augment proliferation of purified

T cells by interrupting the transduction of a negative signal (10). In contrast, whole anti-CTLA-4 mAbs, that could cross-link CTLA-4, inhibited T cell proliferation when the T cells were activated by anti-CD3 in the presence of optimal CD28 costimulation. Monovalent Fab fragments of the antibody, that cannot cross-link CTLA-4, had no effect in this system (10). In addition to the in vitro and in vivo data using mAbs, there is now direct evidence for a physiologic role for CTLA-4 in the downregulation of activated T cells. CTLA-4-deficient mice manifest a massive lymphoproliferative disorder, increased numbers of activated T cells and autoimmune-like tissue destruction (16, 17). These data are consistent with an integral role for CTLA-4 signaling in the negative regulation of activated T cells.

Although there is now ample evidence to suggest that CTLA-4 is involved in the downregulation of activated T cells, the mechanism remains unclear. Two possibilities seem plausible. First, CTLA-4 ligation may inhibit T cell function by inducing apoptosis in activated T cells. In support of this possibility, Gribben et al. (18) have shown that incubation of preactivated human T cells with antigen in the presence of an anti-CTLA-4 mAb induces apoptosis. Second, CTLA-4 may inhibit T cell activation by preventing early or late events in T cell activation, such as gene expression, growth factor production, and/or cell cycle progression. If CTLA-4 is a CD28 antagonist, it is possible that it may function, in particular, to regulate IL-2 production, a growth factor known to be induced by CD28 ligation and to be important for cell cycle progression and T cell expansion.

In the present study, anti-CTLA-4 mAb were used to

further examine the role of CD28 in the regulation of CTLA-4 expression as well as the mechanism by which CTLA-4 inhibits T cell activation. The results demonstrate that CTLA-4 expression on naive T cells is regulated by costimulation through CD28/B7-2 interactions. In addition, antibodies to CTLA-4 inhibit anti-CD3/anti-CD28-induced T cell proliferative responses by blocking IL-2 production, sustained IL-2 receptor expression and events that regulate progression of the cells into the cell cycle. CTLA-4 does not seem to regulate programmed cell death.

## Materials and Methods

**Animals.** 4–8-wk-old C57BL/6 (B6) mice were purchased from Frederick National Cancer Institute (Bethesda, MD). 4–8-wk-old 2C TCR transgenic (2C)<sup>1</sup> mice were derived as previously described (19), bred, and maintained under specific-pathogen free conditions in the University of Chicago Animal Barrier Facility. 2C  $\times$  *lpr/lpr* [2C(*lpr*)] mice were generated by breeding 2C animals to B6.*lpr/lpr* (purchased from the Jackson Laboratory, Bar Harbor, ME). F1 mice were screened for the 2C transgenes with the anti-clonotypic mAb, 1B2 (20) and 2C Tg<sup>+</sup> F1 mice were bred back to B6.*lpr/lpr*. Offspring of this mating were screened for the 2C transgenes as described above and for the *lpr* mutation by polymerase chain reaction (PCR) of tail DNA as described elsewhere (21). 2C(*lpr*) mice were used at 3–5 wk of age.

**Cell Lines and Reagents.** UC10-4F10-11 (hamster anti-murine CTLA-4 [10]), 145-2C11 (hamster anti-murine CD3 [22]), 2.4G2 (rat anti-mouse FcR [23]), 1B2 (mouse anti-2C TCR [20]), J11d (rat anti-murine HSA [24]), GL-1 (rat anti-murine B7-2 [25]), AT83A (rat anti-Thy1.2 [26]), and the 25-9-3 (mouse anti-I-A<sup>b</sup> [27]) were prepared in our laboratory. PV-1 (hamster anti-murine CD28 [28]) was kindly provided by Dr. Carl June (Naval Medical Research Institute, Bethesda, MD). Purified hamster Ig (purchased from Cappel Research Products, Durham, NC) was used as negative control hamster antibodies in *in vitro* cultures. Control human Ig, human CTLA4Ig, 16-10A1 (hamster anti-murine B7-1 [29]) and Fab fragments of the anti-CD28 mAb were provided by the Repligen Corporation (Cambridge, MA). The coding sequence for the extracellular portion of human CTLA-4 was joined to the hinge-CH2-CH3 domains derived from a human genomic IgG<sub>1</sub> gene by PCR as previously described (30). Fab fragments of the anti-CTLA-4 mAb, UC10-4F10-11 were generated in our laboratory as previously described (10). Anti-CTLA-4 Fab fragments were tested in comparison to whole anti-CTLA-4 mAb for their ability to competitively inhibit binding of FITC-conjugated UC10-4F10-11 to CTLA-4 transfected CHO cells (10). The Fab fragments inhibited binding of the FITC-conjugated anti-CTLA-4 ~10-fold less well than whole anti-CTLA-4. However, multiple studies both *in vitro* (10) and *in vivo* (15) have shown that the doses of Fab used in the present studies are sufficient to mediate biological effects. Rat anti-murine CD8 (2.43 [31]) and hamster anti-murine CTLA-4 (UC10-4F10-11 [10]) coupled to FITC were prepared in our laboratory. Biotinylated anti-IL-2R (7D4) was purchased from PharMingen (San Diego, CA). FITC-conjugated anti-CD3 (145-2C11) was provided by Boehringer Mannheim (Indianapolis, IN). Phycoeryth-

rin-conjugated streptavidin (SAV-PE) was purchased from Southern Biotech (Birmingham, AL).

**Bulk T Cell Activation Cultures to Examine CTLA-4 Induction.** Spleens were harvested from B6 mice and depleted of erythrocytes with ammonium chloride-potassium lysing buffer. Splenocytes were cultured alone in DMEM containing 10% fetal calf serum or in the presence of 10  $\mu$ g/ml soluble anti-CD3. To examine the role of CD28 in the regulation of CTLA-4 expression, Fab fragments of anti-CD28, CTLA4Ig, anti-B7-1, anti-B7-2, control human Ig or control hamster Ig were added at 100  $\mu$ g/ml final concentration. Cells were examined for CTLA-4 expression by flow cytometry at 48 h after the initiation of culture.

**Flow Cytometric Analysis.** After harvest, cells were washed once in FACS<sup>®</sup> buffer (0.1% BSA and 0.01% Sodium Azide in PBS, pH 7.4) and simultaneously stained with FITC- and biotin-conjugated mAb as described for 30 min at 4°C. Cells were washed again in FACS<sup>®</sup> buffer and incubated with SAV-PE to develop biotinylated mAb for 15 min at RT. After a final wash in FACS<sup>®</sup> buffer, cells were analyzed on a FACScan<sup>®</sup> flow cytometer (Becton-Dickinson, Mountain View, CA). Incorporation of propidium iodide was used to exclude dead cells. Data was analyzed using both Lysis II (Becton-Dickinson) and WinMDI software (Joseph Trotter, Salk Institute, available by anonymous ftp off flo-sun.salk.edu in /pub/pc).

**T Cell Proliferation Assays.** 2C and 2C(*lpr*) lymph node cells were enriched for T cells by passage over nylon wool columns. MHC Class II<sup>+</sup> cells were further depleted using a mixture of anti-HSA (J11d) and anti-I-A<sup>b</sup> (25-9-3) culture supernatants plus rabbit complement (Pel-Freez, Brown Deer, WI). T cell purity was evaluated by flow cytometry using anti-CD3 mAb. In all cases, T cells used in these assays were >97% CD3<sup>+</sup>. Purified T cells were plated out at  $2 \times 10^5$ /well in the presence of  $1 \times 10^5$  irradiated (2,000 rads) syngeneic, erythrocyte-depleted B6 splenocytes. Syngeneic feeder cells were depleted of T cells by incubation with anti-Thy 1.2 and rabbit complement. Anti-CD3 (0.1  $\mu$ g/ml), anti-CD28 (1  $\mu$ g/ml), anti-CTLA-4 (50  $\mu$ g/ml), control hamster IgG (50  $\mu$ g/ml), anti-CTLA-4 Fab fragments (50  $\mu$ g/ml), and recombinant human IL-2 (rIL-2) (50 U/ml) were used as described in the results section. Assays were carried out for 72 h at 37°C, and were pulsed with 1  $\mu$ Ci/well [<sup>3</sup>H]thymidine (Amersham Corp., Arlington Heights, IL) for the last 16 h of culture. Counts are represented as the mean of triplicate wells with standard errors <10%. All experiments were carried out a minimum of three times with similar results.

**IL-2 Determination.** Supernatants from duplicate cultures of the above described proliferation assays were harvested at 24 h after initiation of culture and stored at -20°C until assayed using a commercial IL-2 ELISA kit from Endogen (Cambridge, MA). A murine IL-2 standard was used to quantify IL-2 levels in the supernatants (represented as pg/ml).

**Terminal Deoxynucleotidyl Transferase (Tdt)-mediated dUTP Nick End Labeling (TUNEL) Assay for Apoptosis.** Cells were harvested 72 h after the initiation of culture and washed once in FACS<sup>®</sup> buffer. Harvested cells were fixed overnight at 4°C in 1% para-formaldehyde. Cells were then washed twice in PBS before incubation in Tdt buffer containing 2.5 mM CoCl<sub>2</sub> (as supplied by Boehringer Mannheim (Indianapolis, IN)). DNA degradation was determined by the level of incorporation of BODIPY-conjugated dUTP (Molecular Probes, Eugene, OR) when Tdt enzyme was added to the fixed cells. Suspended cells were incubated in the dark for 30 min in a 37°C water bath, with or without Tdt, washed once in 15 mM EDTA, once in 0.1% Triton-X 100, and finally suspended in PBS. Cells were analyzed for BODIPY-dUTP in-

<sup>1</sup>Abbreviations used in this paper: 2C, 2C TCR transgenic; B6, C57BL/6; control Ig, control hamster immunoglobulin; FcR, Fc receptor; PCD, programmed cell death; Tg, transgenic; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

corporation by flow cytometry. Logarithmically amplified fluorescence data were collected on  $5 \times 10^3$  cells.

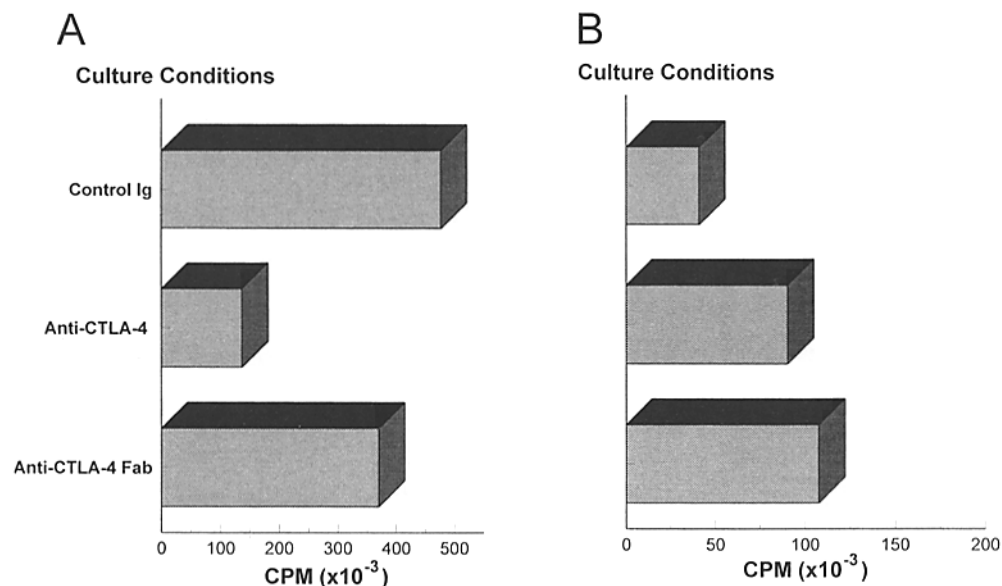
**Cell Cycle Analysis.** Cultured cells were passed over Ficol-Hypaque to remove dead cells. The live cells were stained with anti-CD8-FITC in FACS® buffer, washed in PBS and fixed in 0.25% paraformaldehyde for 15 min at room temperature. Cells were washed once in PBS, fixed with 70% methanol and stored overnight at  $-20^\circ\text{C}$ . After 2 washes in PBS, cells were treated with RNase at  $10 \mu\text{g/ml}$  at  $37^\circ\text{C}$  for 30 min. Cells were washed once in PBS and resuspended in PBS containing  $10 \mu\text{g/ml}$  propidium iodide. Logarithmically amplified fluorescence data was collected on  $10^4$  cells.

## Results

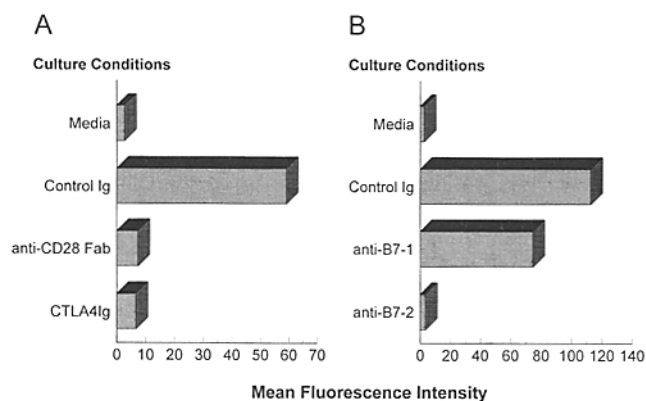
**Anti-CTLA-4 mAb Ig But Not Fab Fragments Inhibit T Cell Proliferative Responses.** As described previously (10), anti-CTLA-4 mAb, when cultured in the presence of anti-CD3 mAb and optimal costimulation via an anti-CD28 mAb, inhibited T cell proliferation. This inhibition of proliferation required FcR cross-linking of the anti-CTLA-4 mAb because Fab fragments of the anti-CTLA-4 mAb had no effect on proliferation in this system (Fig. 1 A). The Fab fragments have previously been shown to effectively bind CTLA-4 and modulate immune responses both in vitro (10) and in vivo (15). Similar results were observed upon stimulation of 2C TCR Tg lymph node cells with alloantigen or the bacterial superantigen staphylococcal enterotoxin B (data not shown). It is unlikely that the anti-CTLA-4 mAb blocked proliferation by competing for FcR-mediated cross-linking of the anti-CD3 or anti-CD28 mAbs since the anti-CTLA-4 mAb did not inhibit anti-CD3-mediated T cell activation (Fig. 1 B). In fact, in multiple experiments (Fig. 1 B and reference 10), anti-CTLA-4 mAbs significantly enhance proliferative responses in the absence of maximal CD28 costimulation. Finally, these results have been confirmed and extended by studies of other investigators using anti-CTLA-4 mAbs directly immobilized on plastic beads with anti-CD3 and anti-CD28 mAbs (11).

**CD28 Signaling Regulates CTLA-4 Upregulation.** Optimal CD28 costimulation was essential for the anti-CTLA-4 mAbs to manifest their inhibitory activity. These results suggested that CTLA-4 expression and/or signaling was dependent on CD28 engagement. Previous studies using CD28-deficient mice have suggested that CD28 costimulation was essential for maximal upregulation of CTLA-4 expression (10). To directly examine the role of CD28/B7 interactions in CTLA-4 upregulation, whole B6 spleen cells were stimulated with anti-CD3 in the presence of anti-CD28 Fab fragments or CTLA4Ig. As seen in Fig. 2 (A) both CD28 antagonists inhibited the upregulation of CTLA-4 on the CD8<sup>+</sup> T cells. Interestingly, the addition of anti-B7-2, not anti-B7-1, inhibited CTLA-4 expression (Fig. 2 B) consistent with previous findings suggesting a primary role of B7-2 in CD28-mediated T cell costimulation. Thus, optimal CTLA-4 expression is dependent on costimulation of T cells through the CD28 receptor. In fact, the requirement for CD28 signals to upregulate CTLA-4 expression after T cell activation may explain, in part, why the inhibitory effects of the anti-CTLA-4 mAb are only observed in the presence of optimal costimulation through CD28 (Fig. 1 B). The enhanced proliferative response observed in the absence of anti-CD28 costimulation may reflect an ability of even small levels of high affinity CTLA-4 to compete for B7 binding by CD28.

**Inhibition of Proliferation by Anti-CTLA-4 in the Presence of Anti-CD28 Is Not Fas-dependent.** Previous studies using human T cells have suggested that the engagement of CTLA-4 inhibits T cell proliferation by inducing apoptosis of the activated T cells (18). Fas-mediated cell death has been proposed to be a major pathway for regulating cell expansion after TCR ligation in several model systems (32). Therefore, the role of Fas in anti-CTLA-4-mediated suppression of proliferation was directly examined using the Fas-deficient *lpr* mouse (33, 34). 2C mice were bred to B6.*lpr/lpr* [2C(*lpr*)]. Tg<sup>+</sup> T cells from these animals, which produce a defective Fas protein that is unable to transduce an apop-



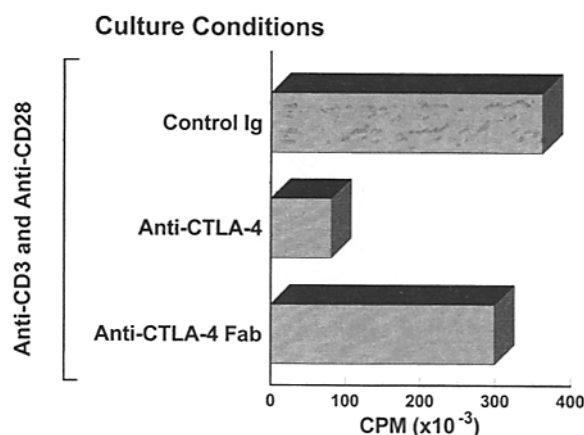
**Figure 1.** Anti-CTLA-4 inhibition of 2C T cell proliferation is CD28-dependent. Two-hundred thousand purified 2C TCR Tg<sup>+</sup> T cells were cultured with  $1 \times 10^5$  syngeneic, irradiated T-depleted splenocytes in the presence of both suboptimal TCR stimulation ( $0.1 \mu\text{g/ml}$  anti-CD3) and optimal co-stimulation ( $1.0 \mu\text{g/ml}$  anti-CD28) (A) or in the presence of anti-CD3 stimulation alone (B). Control Ig, anti-CTLA-4 or anti-CTLA-4 Fab fragments were added at  $50 \mu\text{g/ml}$ . The assay was harvested at 72 h after a 16-h pulse with  $1 \mu\text{Ci/well}$  [ $^3\text{H}$ ]thymidine.



**Figure 2.** Co-stimulation through CD28 regulates CTLA-4 expression. CTLA-4 upregulation was examined in the presence of Fab fragments of anti-CD28 mAb, CTLA4Ig, anti-B7-1 or anti-B7-2 mAb. B6 splenocytes were cultured in the presence of anti-CD3 and 100  $\mu$ g/ml of the indicated antagonist or control Ig for 48 h. CTLA-4 expression was examined on the CD8<sup>+</sup> T cells by two-color flow cytometry. Data represents the mean fluorescence intensity of anti-CTLA-4 staining on the CD8<sup>+</sup> T cell population.

otic signal, were analyzed for their ability to proliferate in response to anti-CD3 and anti-CD28 in the presence/absence of anti-CTLA-4 mAbs. The presence of anti-CTLA-4 mAbs in the activation cultures resulted in profound inhibition of anti-CD3/anti-CD28-induced T cell proliferation (Fig. 3) similar to that observed with control Fas<sup>+</sup> 2C T cells (Fig. 1 A). Together, these results suggested that the mechanism of CTLA-4-mediated effects on T cell proliferation was not via a Fas-dependent apoptotic pathway.

**Inhibition of T Cell Proliferation by Anti-CTLA-4 mAbs Is Not Mediated by Apoptosis.** Recent studies have supported Fas-independent mechanisms of apoptosis after TCR ligation (35). Thus, a TUNEL assay was used to examine whether the anti-CTLA-4 mAbs induced DNA degradation consistent with Fas-independent programmed cell death. As previously shown (5, 10) T cells stimulated with anti-CD3 in the absence of CD28 costimulation exhibit significantly reduced proliferative responses and profound apoptotic death. As seen in Fig. 4, 2C Tg<sup>+</sup> T cells proliferated poorly to anti-CD3 alone (A) and over 40% of the cells were observed to be undergoing apoptosis at 72 h (B). The addition of anti-CD28 mAb significantly increased proliferation and reduced the percentage of cells undergoing apoptosis (6.1–9.5%) (Fig. 4 B). These results are consistent with previous studies demonstrating the cell survival effects of CD28 signaling (5). The effects of anti-CTLA-4 mAbs were examined in this assay. Although the proliferation of activated T cells was significantly inhibited by the addition of the anti-CTLA-4 mAbs (Fig. 4 A), the percentage of apoptotic cells remained comparable to the level observed in control cultures (Fig. 4 B). These data suggested that downregulation of proliferation by CTLA-4 cross-linking was not mediated by induction of apoptosis in activated T cells. Moreover, the results support previous evidence that the anti-CTLA-4 mAb does not mediate its suppressive effects by competing with anti-CD28 for FcR engagement since blockade of CD28 signaling would have been expected to promote apoptosis.

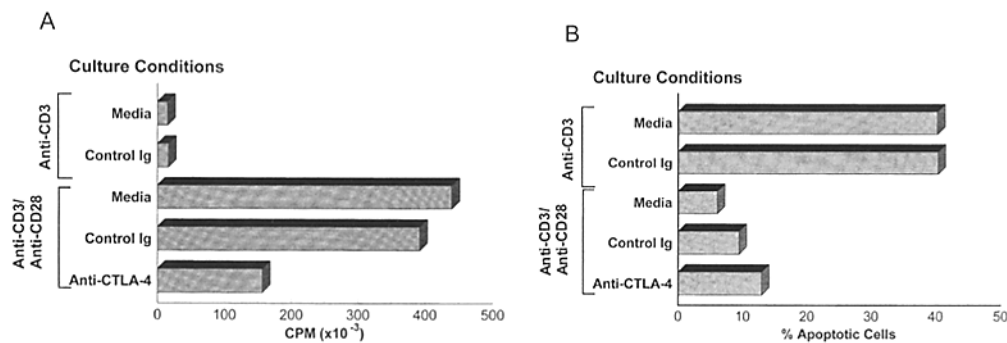


**Figure 3.** Anti-CTLA-4-mediated inhibition of proliferation is Fas-independent. 2C T cell proliferation assays were set up as described in Fig. 1. Responding T cells were purified from the lymph nodes of 2C(lpr) mice. The results depicted in this figure are representative of three separate experiments.

**CTLA-4 Suppresses Late, but Not Early, Cell Cycle Progression.** The previous results suggested that CTLA-4 regulates T cell proliferation by inhibiting proliferation independently of programmed cell death (PCD), perhaps by blocking T cells from progressing through the cell cycle. To address this issue directly, we examined the ability of anti-CD3/anti-CD28-activated T cells to progress through the cell cycle in the presence of anti-CTLA-4 mAbs. As seen in Fig. 5, there was no apparent difference in the progression of T cells into the cell cycle at the early (24 h) time point in anti-CD3/anti-CD28-stimulated T cells cultured in the absence or presence of the anti-CTLA-4 mAbs. In contrast, there were consistently less T cells (15%) in the S/G2-M phase of the cell cycle in the anti-CTLA-4-treated cultures when compared to control Ig-treated cultures at 72 h. As previously noted, CTLA-4 is upregulated after initial T cell activation. Therefore, it is likely that the inhibitory effect of CTLA-4 ligation is subsequent to the initiating signals after TCR ligation that are responsible for driving T cells into early phases of the cell cycle.

**Signals through CTLA-4 Inhibit Sustained Expression of IL-2 Receptor (IL-2R $\alpha$ ) and IL-2 Production.** The previous results suggested that CTLA-4 acts subsequently to the initial TCR signaling events to regulate T cell growth. Therefore, we examined two events known to be important for sustained T cell growth, the expression of the low affinity IL-2R $\alpha$  chain and production of IL-2. Fig. 6 shows the kinetics of IL-2R $\alpha$  chain expression on 2C TCR Tg<sup>+</sup> T cells activated under control conditions or in the presence of anti-CTLA-4 mAbs. Although there was no observable effect of the anti-CTLA-4 mAb on the level of IL-2R $\alpha$  chain expression on the surface of the activated T cells at 24 h, a marked decrease in IL-2R $\alpha$  expression was evident by 48 h (data not shown) and 72 h (Fig. 6). Thus, low affinity IL-2R $\alpha$  chain expression on activated T cells is inhibited by signaling via CTLA-4.

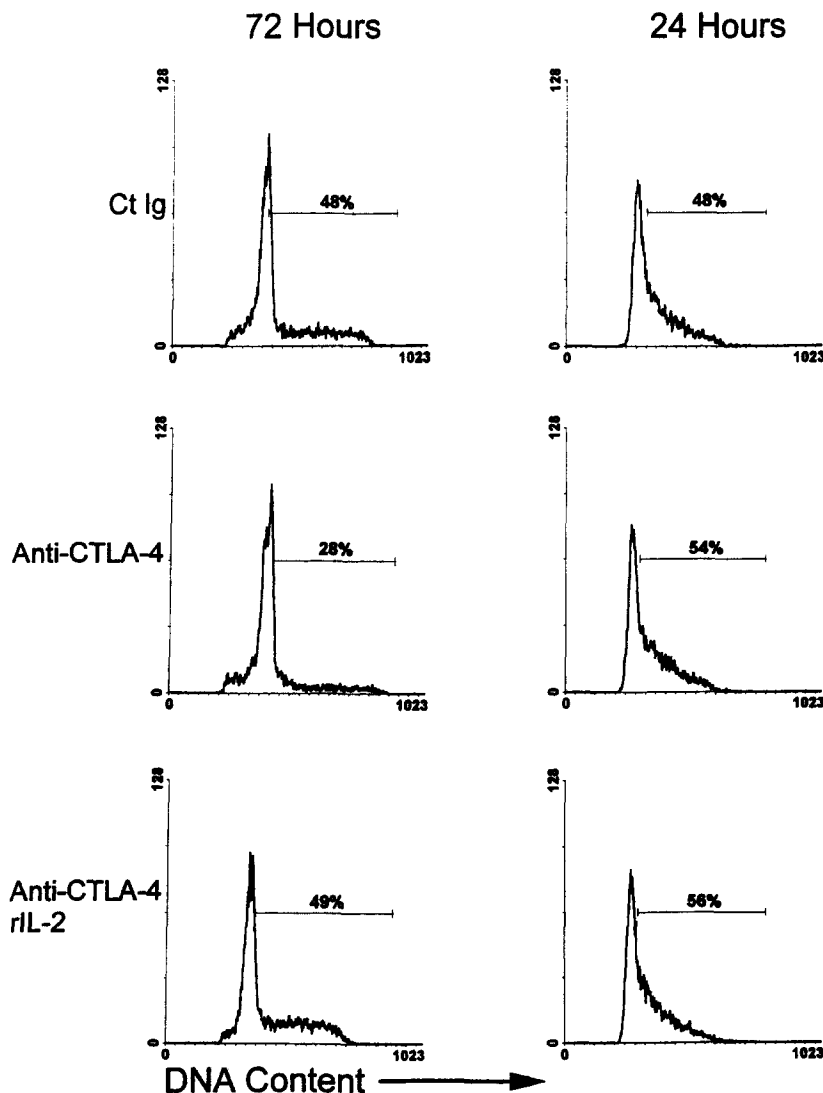
The anti-CTLA-4 mAbs appeared to have a preferential effect on late events in cell cycle progression and IL-2R $\alpha$



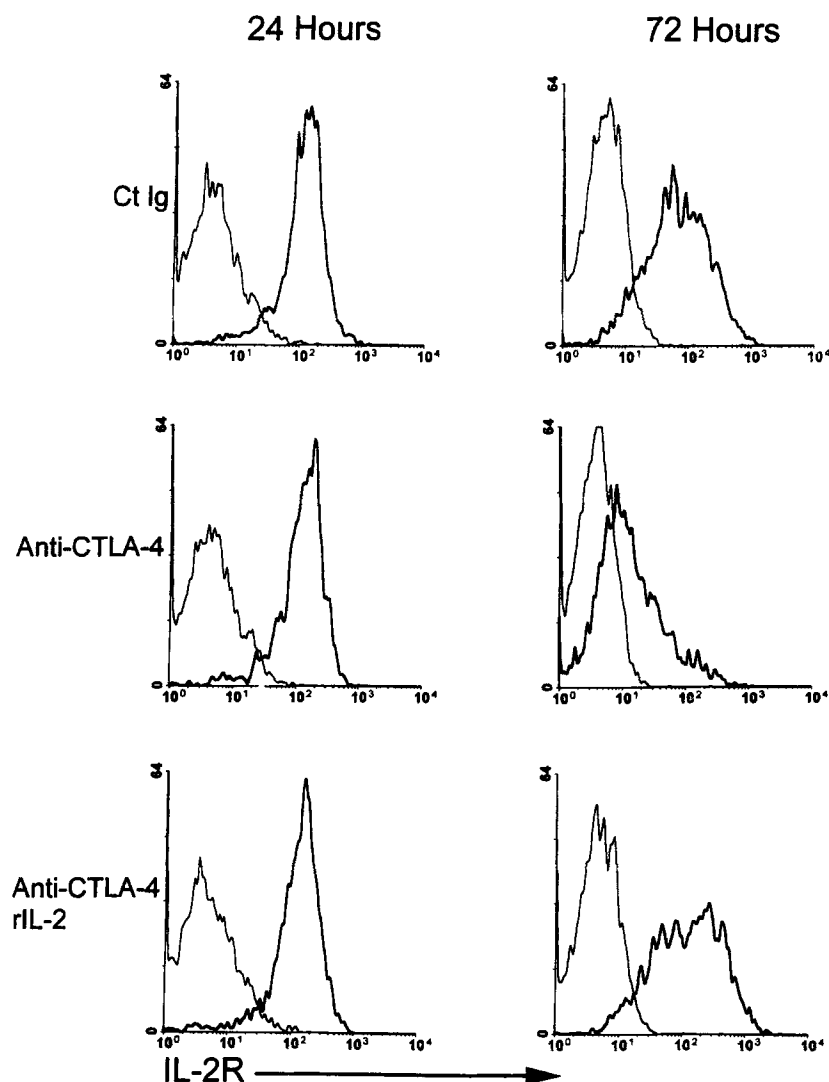
**Figure 4.** Inhibition of proliferation by anti-CTLA-4 mAb is not mediated by apoptosis. The proliferation assay was set up as described in Fig. 1. *A* depicts the proliferative response of 2C T cells subjected to the PCD analyses. *B* depicts the PCD analysis. 2C cells were harvested at 72 h after the initiation of culture. Apoptosis was measured as described in Materials and Methods using the TUNEL assay. The amount of apoptosis was determined by flow cytometry. This assay is representative of three separate experiments.

expression. Previous studies have shown that IL-2 is a positive regulator of IL-2R $\alpha$  expression (36) and is essential for cell cycling, though less is known about the regulation of functional high affinity IL-2R. Therefore IL-2 production by Tg<sup>+</sup> T cells stimulated with anti-CD3/anti-CD28 in the presence of a control hamster IgG or anti-CTLA-4

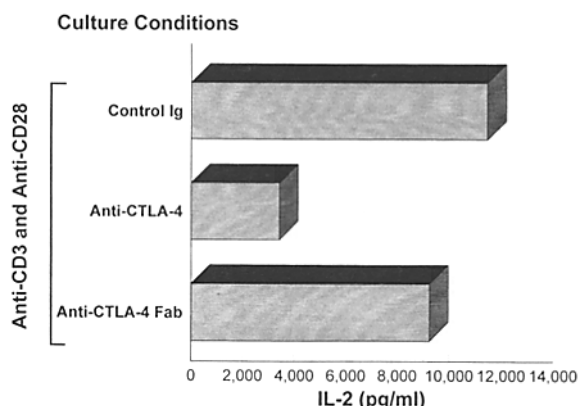
mAb was examined. Significant suppression of IL-2 production (70% in the experiment shown in Fig. 7) by the addition of anti-CTLA-4 mAbs was observed in repeated experiments, consistent with a direct role for CTLA-4 in the regulation of IL-2. In fact, the addition of exogenous IL-2 promoted the aborted cell cycle progression (Fig. 5),



**Figure 5.** CTLA-4 ligation inhibits cell cycle progression. Purified 2C T cells were activated as previously described and harvested at either 24 or 72 h. Cells were stained with CD8-FITC, fixed and DNA staining was performed as described in Materials and Methods. The DNA distribution of CD8<sup>+</sup> T cells is depicted by histograms. The percentage of cells in S/G<sub>2</sub> was determined by comparison of activated cell populations with an unstimulated control 2C T cell population. The top left and right panels represent cells stimulated with anti-CD3/anti-CD28 in the presence of control Ig. The middle left and right panels represent cells stimulated with anti-CD3/anti-CD28 in the presence of anti-CTLA-4 mAbs. The bottom left and right panels represent cells stimulated with anti-CD3/anti-CD28 and anti-CTLA-4 mAb in the presence of exogenous human recombinant IL-2 (50 U/ml).



**Figure 6.** Sustained IL-2 receptor expression is inhibited by anti-CTLA-4 mAb. Purified 2C T cells were stimulated as previously described in Fig. 1 with anti-CD3 and anti-CD28 in the presence of control hamster Ig (*top*), anti-CTLA-4 (*middle*), or anti-CTLA-4 plus IL-2 (*bottom*). The 2C T cells were harvested at 24 and 72 h after activation and examined for IL-2R expression by two-color flow cytometry. The data is represented as histograms for IL-2R expression after gating on CD8<sup>+</sup> T cells.



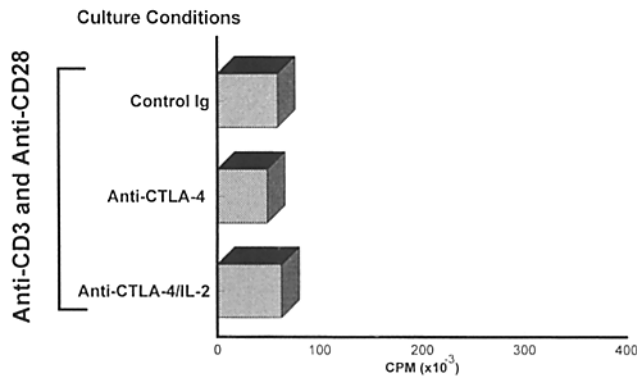
**Figure 7.** CTLA-4 ligation in the presence of CD28 costimulation inhibits the production of IL-2. Purified 2C T cells were stimulated with anti-CD3 (0.1  $\mu$ g/ml) or anti-CD3 and anti-CD28 (1  $\mu$ g/ml) in the presence of Control Ig, anti-CTLA-4, or anti-CTLA-4 Fab fragments (50  $\mu$ g/ml) in the presence of T cell depleted, syngeneic irradiated feeder cells. Supernatants were collected at 24 h and tested for the presence of IL-2 by ELISA.

reversed the downregulation of IL-2R $\alpha$  expression (Fig. 6) and reconstituted T cell proliferation (Fig. 8) in the anti-CTLA-4 treated cultures. Together these data suggest that CTLA-4 acts to suppress IL-2 production, resulting in decreased IL-2R $\alpha$  expression, but not IL-2 responsiveness, under culture conditions where there is optimal costimulation through CD28.

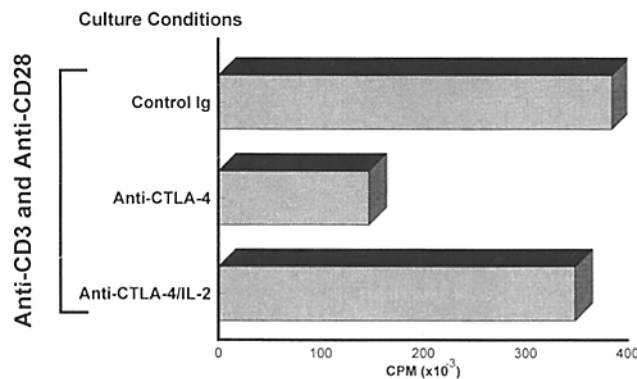
## Discussion

The function of CTLA-4, a cell surface molecule on activated T cells (originally identified in a mRNA library of activated CTL), remains controversial. CTLA-4 was shown to bind B7-1/B7-2 and, based on its sequence homology to the CD28 molecule, was predicted to function as a member of the CD28 class of costimulatory molecules. In fact, early studies supported a costimulatory role for CTLA-4 in promoting T cell proliferation (9, 37). However, more re-

## 24 Hours



## 72 Hours



**Figure 8.** Inhibition of proliferation by anti-CTLA-4 mAb can be reversed by the addition of exogenous IL-2. Purified 2C T cells were stimulated with anti-CD3 and anti-CD28 (as described in Fig. 1) in the presence of Control Ig, anti-CTLA-4 mAb or anti-CTLA-4 mAb and recombinant human IL-2 (50 U/ml) (Anti-CTLA-4/IL-2). Proliferation was measured at both 24 and 72 h after the initiation of culture.

cent studies have suggested that rather than promoting T cell activation, engagement of CTLA-4 actually inhibits T cell function (10, 11, 18). First, we showed that blockade of CTLA-4 engagement with its natural ligands by Fab fragments of anti-CTLA-4 mAbs enhanced T cell proliferation both in vitro and in vivo (10, 15). In addition, we, and others, developed an assay system wherein cross-linking CTLA-4 with mAbs inhibited T cells stimulated through CD3 and CD28 (10, 11). Finally, recent studies using CTLA-4-deficient mice have emphasized the importance of the CTLA-4-mediated downregulatory activity. CTLA-4 knockout mice demonstrate a massive lymphoproliferative response (16, 17). In the present studies, we address the mechanism by which CTLA-4 downregulates T cell function. The data suggest that CTLA-4 does not mediate its effects through Fas-mediated or other forms of programmed cell death. However, CTLA-4 cross-linking in-

hibits cell cycle progression, sustained IL-2R $\alpha$  expression and IL-2 production. Furthermore, the most profound effects of CTLA-4 cross-linking occur in the presence of maximal CD28-mediated T cell costimulation.

There are several possible models for how CTLA-4 may be antagonizing CD28-mediated signals. It is possible that CTLA-4 may inhibit T cell activation by directly antagonizing initial CD28 signals, especially since CTLA-4 has a higher affinity for B7-1 and B7-2 than does CD28 (7, 38). This "cell surface competition" hypothesis does not fully explain the results for two reasons. First, we have observed opposing effects of whole versus Fab fragments of the anti-CTLA-4 mAbs. The addition of whole anti-CTLA-4 mAb inhibited T cell activation when anti-CD3 and anti-CD28 were present, while the addition of Fab fragments to the culture had no effect or actually enhanced T cell proliferation. If CTLA-4 was merely competing with CD28 for interaction with the B7 family members, both Fab fragments and whole anti-CTLA-4 mAbs would be expected to augment proliferation by blocking CTLA-4 interactions with the counter-receptors, thus making more of the B7-family member molecules available for CD28 ligation. Second, if CTLA-4 was competing with CD28 for B7 ligation, then CD28 signaling would have been expected to be impaired on the naive T cells. It has previously been shown that the blockade of CD28/B7 interactions leads to a rapid decrease in cell survival as a direct result of apoptosis (5). The present study showed that inhibition of T cell proliferation after CTLA-4 cross-linking was not related to apoptosis, although we cannot rule out that rapid apoptosis of cells might not be detected in these cultures. However, overall cell yield over time would not be consistent with this interpretation of the data. Analyses of Fas-defective T cells failed to provide any evidence of increased cell death among T cells cultured with the anti-CTLA-4 mAb, nor did the mAb affect augmentation of cell survival effected by anti-CD28. Gribben et al. (18) have suggested that CTLA-4 may regulate T cell function by inducing apoptosis of activated T cells, since, in their study, one anti-CTLA-4 mAb was shown to directly mediate apoptosis of human alloreactive T cell clones. There are two important differences that may explain the differences between our study and those of Gribben et al. First, the anti-CTLA-4 mAb described does not react with the same epitope as UC10-4F10-11. UC10-4F10-11 inhibits the interaction of B7-1 or B7-2 with murine CTLA-4Ig (data not shown). In contrast, the anti-human CTLA-4 mAb studied by Gribben et al. (18) was selected for its distinct binding to a CTLA-4 epitope unrelated to B7 binding. In fact, anti-CTLA-4 antibodies that interacted with the B7 binding site did not induce apoptosis in their system. Second, our studies used primary, naive T cells as opposed to T cell clones. Since T cell clones are dependent ultimately on IL-2 for survival, it is possible that inhibition of IL-2 production by the T cell clones led to the observed programmed cell death in the Gribben et al. study (18).

The finding that anti-CTLA-4 mAbs appeared to preferentially affect IL-2 production early following T cell activa-



tion is intriguing. Several early T cell activation events such as entry into cell cycle and initial IL-2R upregulation were not altered by CTLA-4 cross-linking. However, IL-2 production was inhibited at the 24-h time point. Lucas et al. recently reported similar results documenting that IL-2 production, but not early IL-2R expression, was severely impaired in CD28 knockout mice (39). In addition, Sperling et al., (Sperling, A.I., J.A. Auger, B. Ehst, I.C. Rulifson, C.B. Thompson, and J.A. Bluestone, manuscript submitted for publication) have shown that CD28 signals may not be required for early T cell activation events, but are necessary for T cell survival and sustained proliferative responses. In fact, it is striking that the most profound inhibition of T cell activation following TCR cross-linking occurs in the presence of optimal costimulation through CD28. These results suggest that cross-linking by anti-CTLA-4 mAbs selectively inhibits CD28-dependent signaling events. The data also support recent suggestions that IL-2 production is not essential to initiate the transit of T cells into the cell cycle, but that the lack of this growth factor may impede cell cycle progression, prevent sustained IL-2R expression and T cell expansion.

Thus, it will be important to determine whether the negative signals delivered by CTLA-4 cross-linking directly antagonize signals through CD28 (such as the activation of protein tyrosine kinases) that are essential for IL-2 mRNA induction or regulate signals (such as TCR signals) independent of those mediated by CD28. For instance, CTLA-4 and CD28 may share common signaling pathways and initiation of CTLA-4 signaling may antagonize CD28 signaling events by preventing the interaction of CD28 with necessary cytoplasmic signaling molecules, perhaps by alter-

ing phosphorylation events. As an example, it has been shown that both CD28 and CTLA-4 can bind and activate PI-3 kinase (40). However, CTLA-4 ligation may regulate PI-3 kinase activity. Alternatively, CTLA-4 cross-linking may activate biochemical pathways that antagonize signals initiated by CD28. CD28 has been shown to regulate IL-2 at the level of IL-2 gene transcription and mRNA stability (41–43). Thus, CTLA-4 signals may regulate IL-2 secretion by inhibiting the induction of specific IL-2 transcription factors or affect posttranscriptional stability of IL-2 mRNA.

Finally, it is difficult to determine from the present studies how early CTLA-4 acts in blocking T cell activation. The present findings suggest that CTLA-4 downregulates naive T cell activation subsequent to CD28 signaling. Unlike CD28, little CTLA-4 is detectable on naive T cells. Rather, CTLA-4 is upregulated after T cell activation, peaking on the cell surface at 48–72 h. Moreover, CTLA-4 upregulation is CD28-dependent, as blockade of CD28 ligation inhibits CTLA-4 induction while increased signaling through CD28 increases CTLA-4 expression on the cell surface (data not shown). Thus, CTLA-4 seems likely to function subsequently to initial T cell activation events. However, it remains possible that some T cells, perhaps memory or recently activated T cells, may express low levels of CTLA-4 on the cell surface. Under these circumstances CTLA-4 engagement may block the initiation of T cell activation. In either case, it has become increasingly clear that CTLA-4 serves a role as a major repressor of the immune response. Future studies will focus on manipulating this downregulatory function to either enhance suboptimal immune responses in cancer and infectious disease or block activated T cells in transplantation and autoimmunity.

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Address correspondence to Jeffrey Bluestone, Ben May Institute for Cancer Research, MC1089, University of Chicago, 5841 S. Maryland, Chicago, IL 60637.

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